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# Polarized Membrane Traffic and Cell Polarity Development Is Dependent on Dihydroceramide Synthase-Regulated Sphinganine Turnover

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Sphingoid bases have been implicated in various cellular processes including cell growth, apoptosis and cell differentiation. Here, we show that the regulated turnover of sphingoid bases is crucial for cell polarity development, i.e., the biogenesis of apical plasma membrane domains, in well-differentiated hepatic cells. Thus, inhibition of dihydroceramide synthase or sphinganine kinase activity with fumonisin B1 or *N,N*-dimethylsphingosine, respectively, dramatically perturbs cell polarity development, which is due to increased levels of sphinganine. Consistently, reduction of free sphinganine levels stimulates cell polarity development. Moreover, dihydroceramide synthase, the predominant enzyme responsible for sphinganine turnover, is a target for cell polarity stimulating cAMP/protein kinase A (PKA) signaling cascades. Indeed, electrospray ionization tandem mass spectrometry analyses revealed a significant reduction in sphinganine levels in cAMP/PKA-stimulated cells. These data suggest that sphinganine turnover is critical for and is actively regulated during HepG2 cell polarity development. Previously, we have identified an apical plasma membrane-directed trafficking pathway from the subapical compartment. This transport pathway, which is part of the basolateral-to-apical transcytotic itinerary, plays a crucial role in apical plasma membrane biogenesis. Here, we show that, as a part of the underlying mechanism, the inhibition of dihydroceramide synthase activity and ensuing increased sphinganine levels specifically perturb the activation of this particular pathway in the de novo apical membrane biogenesis.

## INTRODUCTION

Epithelial cell polarity depends on the structural asymmetry of apical and basolateral plasma membrane (PM) domains, separated by junctional complexes. The establishment and maintenance of PM domains requires a careful orchestration of extra- and intracellular signals, triggering crucial organelle- and membrane traffic-linked machineries (Yeaman *et al.*, 1999; Mostov *et al.*, 2003). Hepatocytes, like all epithelia, display distinct apical, bile canalicular (BC) and basolateral, sinusoidal PM domains. Some of the intracellular sites and molecular components of the mechanisms that contribute to apical BC biogenesis in hepatocytes have been clarified. These include E-cadherin (Terry and Gallin, 1994; Matsui *et al.*, 2002), cAMP/protein kinase (PK)A and PKC activities (Roelofs *et al.*, 1998; Zegers and Hoekstra, 1998), the cytoskeleton (Mishra *et al.*, 1999), and the sorting of specific sphingolipids and proteins, mediated by the Golgi and endosomal recycling system (Zegers and Hoekstra, 1998; Ait Slimane and Hoekstra, 2002; Maier and Hoekstra, 2003). In particular, the subapical compartment (SAC), the

hepatocyte equivalent of the common endosome in other epithelia, plays a central role in the polarized trafficking of proteins (Ihrke *et al.*, 1998; van IJzendoorn *et al.*, 1998; Rahner *et al.*, 2000; Silver *et al.*, 2001; Wüstner *et al.*, 2004), sphingolipids (van IJzendoorn and Hoekstra, 1998, 1999b, 2000; Maier and Hoekstra, 2003), and cholesterol (Silver *et al.*, 2001; Wüstner *et al.*, 2001, 2002, 2004).

Studies of the intracellular flow of fluorescent lipid analogs indicated the existence of multiple membrane domains in SAC, and the membrane sorting capacity of the SAC was demonstrated to be a target for signals that promote apical-basolateral PM asymmetry, including cAMP/PKA and the interleukin-6 family cytokine oncostatin M (van IJzendoorn *et al.*, 2000; van der Wouden *et al.*, 2002). Thus, during cell polarity development and/or upon stimulation of cAMP/PKA signaling in well-differentiated hepatic HepG2 cells, a specific SAC-to-BC pathway followed by transcytosing IgA and two fluorescent sphingolipid analogs, C<sub>6</sub>-NBD-sphingomyelin (SM) and -galactosylceramide (GalCer), and distinct from the apical PM recycling route marked by the flow of C<sub>6</sub>-NBD-glucosylceramide (GlcCer), is activated. Blocking this pathway, e.g., with PKA inhibitors or the microtubule-disrupting agent nocodazole effectively perturbs polarity development. After the establishment of apical-basolateral cell polarity, the trafficking of C<sub>6</sub>-NBD-SM and -GalCer from SAC switches from an apical to a basolateral destination, whereas the apical recycling of C<sub>6</sub>-NBD-GlcCer remains unaffected. Fluorescent lipid analogs are thus a useful tool in living cells to monitor intracellular trafficking pathways, and

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alterations in these, that are relevant for cell polarity development.

Sphingolipids, together with cholesterol, cluster to form particularly ordered membrane environments or rafts, in which apical proteins and/or signal transduction molecules are coassembled (Simons and Ikonen, 1997; Ait Slimane *et al.*, 2003). Therefore, the lateral dynamics and organization of sphingolipids may play a prominent role in the regulation of signaling cascades and cell polarity (Holthuis *et al.*, 2001). Indeed, the segregation of predominantly apical PM proteins into sphingolipid-enriched rafts suggests a prominent role for sphingolipids in apical sorting (Simons and Ikonen, 1997). Moreover, the timely regulated activity of enzymes in the sphingolipid biosynthesis pathway is crucial for cellular development, e.g., that of polarized neurons (Futerman, 1998; Schwarz and Futerman, 1998; Ledesma *et al.*, 1999; Boldin and Futerman, 2000). For example, the expression of sphingomyelin in developing neurons coincides with the segregation of Thy-1 into sphingolipid rafts and its subsequent directed trafficking to the axonal plasma membrane domain (Ledesma *et al.*, 1999).

In addition to achieving specific expression levels of complex (glyco)sphingolipids such as SM, GlcCer, and GalCer, the regulated activity of enzymes that control their biosynthesis also may serve to control the levels and activity of its precursors. Indeed, ceramide (CER) and the sphingoid base backbones of sphingolipids, i.e., sphingosine and sphinganine (dihydro-sphingosine), seem highly bioactive compounds that mediate membrane second messenger cascades in the regulation of cell proliferation, apoptosis, and differentiation (Shayman, 2000; Hannun *et al.*, 2001) and, as recently reported, membrane traffic (Friant *et al.*, 2000, 2001; Zanolari *et al.*, 2000). Down-regulation of the expression levels of sphingoid bases is typically achieved by incorporating these in higher sphingolipids or by their phosphorylation by sphingosine/sphinganine kinase. Not surprisingly, alterations in the cellular levels of free sphingoid bases have been implicated in various diseases, including hepatocellular carcinogenesis (Spiegel and Merrill, 1996), which arises from dedifferentiation of mature hepatocytes (Bralet *et al.*, 2002; Gournay *et al.*, 2002). However, no data are available as to the involvement of free sphingoid bases in cell polarity development. In this study, we demonstrate that the predominant enzyme involved in sphingoid base turnover, dihydroceramide synthase (sphinganine *N*-acyltransferase), is a target for cell polarity-stimulating signaling cascades and that the ability of cells to metabolize sphinganine is essential for regulated polarized membrane trafficking from the SAC and cell polarity development.

## MATERIALS AND METHODS

Hoechst-33250 (bisbenzimidazole), L-cycloserine, and Fumonisin B1 were obtained from Sigma-Aldrich (St. Louis, MO). *D*-erythro-Sphinganine, *L*-threo-sphinganine (safingol), *D*-erythro-sphinganine-1-phosphate, and *N,N*-Dimethyl-*D*-erythro-sphingosine were from Avanti Polar Lipids (Alabaster, AL). Dibutyl cAMP was purchased from Roche Diagnostics (Mannheim, Germany). Albumin (from bovine serum, fraction V) was bought from Fluka (Buchs, Switzerland). 6-[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]hexanoic acid (*C*<sub>6</sub>-NBD) and Alexa594-labeled phalloidin were purchased from Molecular Probes (Eugene, OR). Sodium dithionite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>) was from Merck (Darmstadt, Germany). *C*<sub>6</sub>-NBD-sphingolipids were synthesized as described previously (Kishimoto, 1975; Babia *et al.*, 1994). Texas Red (TxR)-labeled IgA was kindly provided by Dr. K. Dunn (Indiana University Medical Center, Indianapolis, IN). All other chemicals were of analytical grade.

### Cell Culture

HepG2 cells were cultured in high glucose (4500 mg/l) DMEM, supplemented with 10% heat-inactivated fetal calf serum (56°C; 30 min), L-glutamine, and antibiotics (penicillin/streptomycin) at 37°C in a 5% CO<sub>2</sub>-humid-

ified incubator, as described previously (van IJzendoorn and Hoekstra, 1998). Cells were plated onto ethanol-sterilized glass coverslips and cultured for 3 d for cell polarity to develop. Cell polarity development in HepG2 cells is characterized by the appearance of microvilli-lined apical vacuoles (resembling BC) that occur in between two adjacent cells (Zegers and Hoekstra, 1997). The establishment of HepG2 cells that stably express the polymeric Ig receptor has been described previously (van IJzendoorn and Hoekstra, 1998).

### Determination of HepG2 Cell Polarity Development

HepG2 cell polarity development was determined by fluorescent microscopy (AX70; Olympus, Tokyo, Japan) analysis of the ratio BC/100 cells as described previously (van IJzendoorn and Hoekstra, 1999, 2000). BC were identified by the dense F-actin staining (Alexa594-labeled phalloidin) that surrounds the BC, and DNA was stained with Hoechst to facilitate cell counting. Identical results were obtained when BC were identified by immunofluorescent labeling of resident apical proteins such as MDR1, DPPIV, or APN (Ait Slimane *et al.*, 2003). Data are expressed as mean  $\pm$  SEM of at least three independent experiments, carried out in duplicate.

### Quantification of Cellular Sphingolipid Levels by Tandem Mass Spectrometry

Ceramide species were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) by the principle described previously with some modifications (Liebisch *et al.*, 1999). In brief, CER was analyzed by direct flow injection analysis using an HTS PAL autosampler (Zwingen, Switzerland) and an Agilent 1100 binary pump (Waldbronn, Germany), providing a constant flow of methanol 10 mM ammonium acetate:chloroform (3:1). The CER-specific daughter ion of *m/z* 264 was used for mass spectrometric detection (Quattro Ultima; Micromass, Manchester, United Kingdom). Quantification was achieved by addition of naturally occurring CER species by using CER 17:0 as a not naturally occurring internal standard. (In a similar way glucosylceramide was measured by the collision-induced generation of *m/z* 264).

Sphingomyelin was quantified by a parent scan of *m/z* 184 and using no naturally occurring phosphatidylcholine (PC) species PC 28:0 and PC 44:0 as internal standards (Brügger *et al.*, 1997). Overlapping PC and SM isotope species were corrected by a self-programmed isotope correction algorithm based on calculated isotope distributions. The detailed methodology will be published in a separate manuscript.

Sphingosine and sphinganine were quantified as described previously (Lieser *et al.*, 2003) by using the instrumentation described above, except for that SDS lysates were used. An effect of the SDS lysis on quantification was excluded because the calibration lines were made in the same sample matrix, which will compensate any possible effect on the analytical response due to the use of SDS. Moreover, additional peaks resulting from the SDS were not observed.

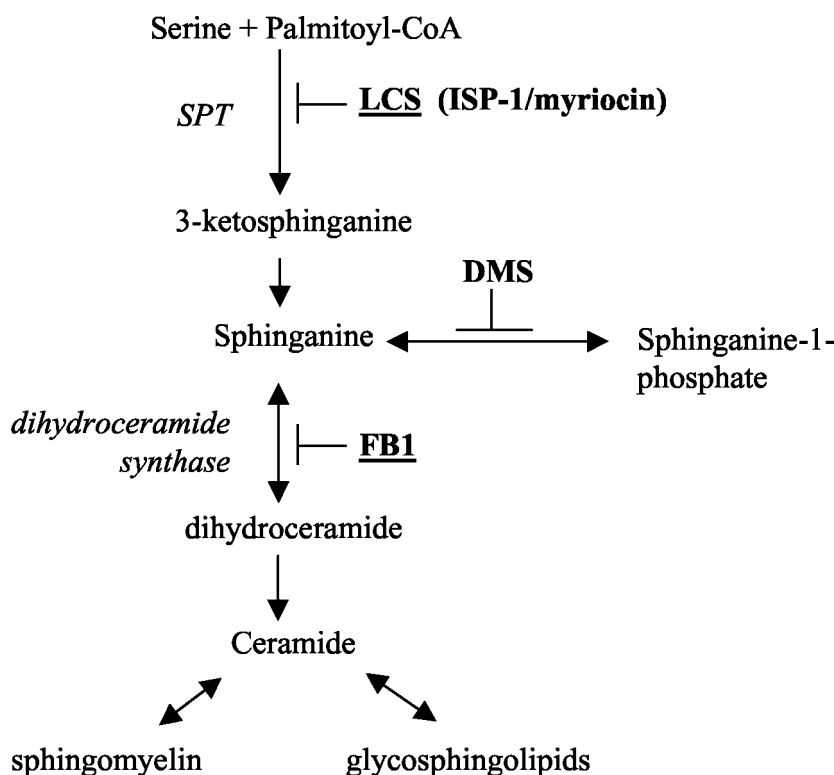
In all experiments a combination of MassLynx software, including the NeoLynx tool (Micromass) and self-programmed Excel macros was used for automated data processing. All data are expressed as mean  $\pm$  SEM of triplicate experiments.

### Dihydroceramide (DHC) Synthase Assay

The dihydroceramide synthase assay was performed as described by Merrill and Wang (1992). Cells, control or treated with dibutyl cAMP (dbcAMP) for 30 min, were scraped, and homogenized in sucrose buffer by sonication. DHC synthase activity was measured in  $\sim 500 \times 10^3$  cells ( $\sim 100 \mu\text{g}$ ) by using 1  $\mu\text{M}$  (100 pmol/100  $\mu\text{g}$  of cell protein) [<sup>3</sup>H]sphinganine and 100  $\mu\text{M}$  stearyl-CoA as substrate in the presence or absence of 10  $\mu\text{M}$  FB1 for 0 or 10 min at 37°C. As an additional control, stearyl-CoA was omitted from the reaction. The reaction was terminated with CHCl<sub>3</sub>:methanol [1:2 (vol/vol)], and lipids were extracted with CHCl<sub>3</sub>:methanol and separated by thin-layer chromatography (TLC). Radiolabeled sphinganine and (dihydro)ceramides were scraped from the TLC plates and quantified in a liquid scintillation counter (Veldman *et al.*, 1998). Data are expressed as picomoles per 100  $\mu\text{g}$  of protein and as percentage of control.

### Transport of *C*<sub>6</sub>-NBD-GalCer from the SAC

To monitor SAC-associated sphingolipid transport, lipid is first accumulated in the SAC, according to a procedure described elsewhere (van IJzendoorn and Hoekstra 1998, 2000). First, cells were washed with PBS and incubated with *C*<sub>6</sub>-NBD-GalCer at 37°C for 30 min to allow internalization of the lipid analogue from the basolateral surface and transcytosis (van IJzendoorn and Hoekstra, 1998). The remaining basolateral pool of lipid analogue was then depleted by a back-exchange procedure [5% (wt/vol) bovine serum albumin in Hanks' balanced salt solution (HBSS), pH 7.4, at 4°C twice for 30 min]. Then the lipid was chased from the apical, bile canalicular PM into the SAC by an incubation at 18°C in back-exchange medium. The chase was done over a 60-min period and at this time, the majority of the lipid analogue was associated with the SAC (cf. van IJzendoorn and Hoekstra, 1998). Any NBD fluorescence still remaining at the luminal leaflet of the apical PM after the 60-min chase was subsequently abolished by incubating the cells with 30 mM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> at 4°C, a condition that precludes intracellular access of the quencher. After 10 min, Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> was then removed by extensive washing of the cells with ice-cold HBSS. In some experiments, cells were then treated



**Figure 1.** Scheme of the sphingolipid metabolic pathway and sites of action of the distinct sphingolipid synthesis inhibitors. FB1, LCS, and DMS inhibit the activity of dihydroceramide synthase, serine palmitoyl transferase, and sphingosine/sphinganine kinase, respectively.

with dbcAMP and/or sphinganine at 4°C for 30 min. Transport of the lipid analogue from the SAC was subsequently monitored by incubation in back-exchange medium at 37°C. When required, dbcAMP and/or sphinganine were kept present during the transport assay.

To quantitate transport of the lipid analogue to and from the apical, BC membranes, the percentage of NBD-positive BC membranes was determined as described previously (van IJzendoorn *et al.*, 1997; van IJzendoorn and Hoekstra, 1998). Thus, BC were first identified by phase-contrast illumination and then classified as either NBD positive or NBD negative under epifluorescence illumination. Distinct pools of fluorescence were discerned, present in vesicular structures adjacent to BC, which have been defined as SAC (cf. van IJzendoorn and Hoekstra, 1998). Together, BC and SAC thus constitute the bile canicular, apical pole (BCP) in HepG2 cells. Therefore, within the BCP region the localization of the fluorescent lipid analogues will be defined as being derived from BC, SAC, or both. This also provides a means to describe the movement of the lipid within or out of this region in the cell. Thus, after loading the SAC with lipid analogue and a subsequent incubation as described above, the direction of movement of the lipid analogue from or within the BCP region is inferred from determining the fraction of NBD-labeled BCP (i.e., label in either the BC or SAC or both) at a given time, relative to that labeled when starting the chase ( $t = 0$ ). At least 50 BCPs per coverslip were analyzed. Data are expressed as the mean  $\pm$  SEM of at least three independent experiments, carried out in duplicate.

### Internalization of Texas Red-labeled IgA

HepG2 cells that stably express pIgR were washed and asialoglycoprotein receptors were saturated with excess asialofetuin at 37°C for 30 min to prevent uptake of IgA via these receptors (cf. van IJzendoorn and Hoekstra, 1998). Cells, treated with sphinganine (2  $\mu$ M; 37°C for 1 h) were incubated with TxR-IgA (50  $\mu$ g/ml) at 18°C for 60 min. Cells were then washed to remove nonbound TxR-IgA and further incubated at 37°C for 30 min in the presence or absence of sphinganine.

## RESULTS

### Sphinganine Accumulation Blocks Polarity Development

To examine the involvement of sphingolipid metabolites in the process of hepatocyte polarity development, we used the mycotoxin fumonisin B1 (FB1), which specifically inhibits DHC synthase activity in the sphingolipid biosynthetic pathway (Figure 1). Quantification of cellular sphingolipids

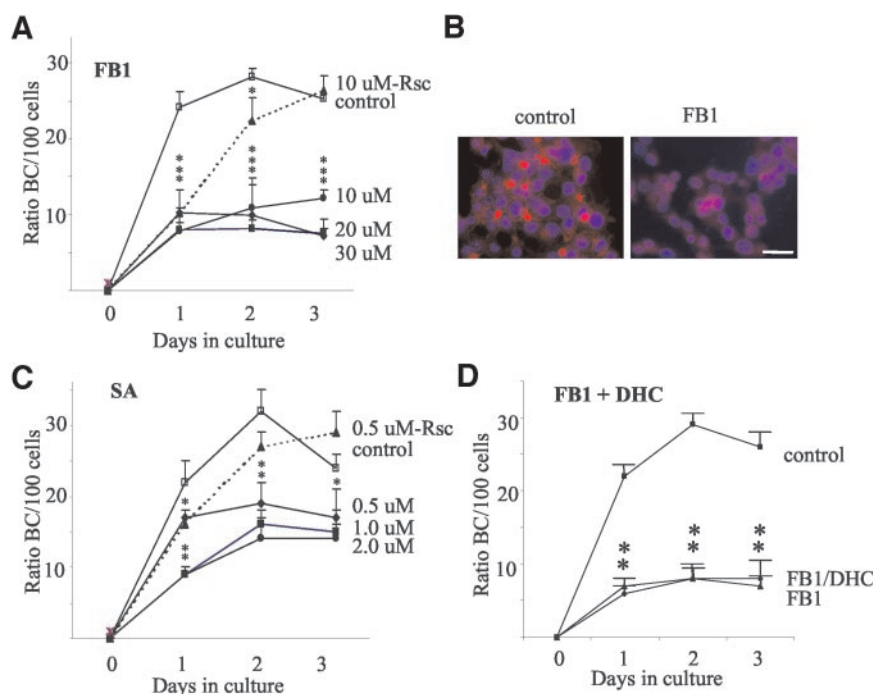
and sphingoid bases was performed by ESI-MS/MS as described in *Materials and Methods* and in Lieser *et al.* (2003). As anticipated, treatment of well-differentiated hepatoma HepG2 cells with FB1 (10  $\mu$ M; 24 h) resulted in decreased levels of ceramides, sphingomyelin, and glucosylceramide (Table 1). Consequently, the level of the sphingoid base sphinganine increased more than fivefold ( $4.1 \pm 1.0$ – $23.0 \pm 12.0$  pmol/mg cell protein; Table 1; Lieser *et al.*, 2003). To examine potential consequences on HepG2 cell polarity development, i.e., the biogenesis of apical BC, cells were plated on coverslips in the presence of 0–30  $\mu$ M FB1. Each day, a coverslip was taken and the ratio BC/100 cells was determined as a measure for cell polarity (see *Materials and Methods*). Control HepG2 cells rapidly acquired polarity, reaching

**Table 1.** Quantification of sphingolipids in control versus FB1-treated cells

	Control (nmol/mg protein)	FB1 (nmol/mg protein)
SM	$21.9 \pm 5.4$	$8.7 \pm 0.8$
GlcCer	$0.09 \pm 0.02$	$0.03 \pm 0.02$
Cer	$0.9 \pm 0.2$	$0.2 \pm 0.03$
SA	$0.0041 \pm 0.001$	$0.023 \pm 0.012$
SO	$0.042 \pm 0.01$	$0.016 \pm 0.004$

Cells were cultured in normal culture medium in the presence of 10  $\mu$ M FB1 or buffer (control) for 24 h. Cells were then lysed in 0.5% SDS lysis buffer, and lysates were immediately frozen at  $-20^{\circ}\text{C}$ . The protein content of each sample was determined, and SM, GlcCer, Cer, sphinganine (SA), and sphingosine (SO) were quantified as described in *Materials and Methods*. All data are expressed as mean  $\pm$  SEM nanomoles per milligram of total cell protein of triplicate experiments ( $n = 3$ ).





**Figure 2.** Accumulation of sphinganine inhibits HepG2 cell polarity development. Cells were treated with different concentrations of FB1, FB1 + DHC, or exogenous *D-erythro*-sphinganine (SA) in serum-supplemented cell culture medium for 24, 48, or 72 h. At each time point, the cells were fixed and the ratio BC/100 cells was determined as described in *Materials and Methods*. Effect of 0–30  $\mu$ M FB1 (A and B), 0.5–2.0  $\mu$ M sphinganine (SA; C), or 30  $\mu$ M FB1 + 10  $\mu$ M DHC (D) on cell polarity development. The dashed lines in A and C (–Rsc) show the course of polarity development after removal of FB1 or exogenous sphinganine, respectively, after 24 h. Data are expressed as mean  $\pm$  SEM of at least three independent experiments carried out in duplicate. (B) Fluorescence microscopical evaluation of cell polarity in control or FB1-treated cells (72 h; nuclei in blue and BC in red). Note the absence of BC in FB1-treated cultures. Bars, 5  $\mu$ m; \* $p$  < 0.05 (Student's *t* test).

a maximum ratio BC/100 cells 2 d after plating (Figure 2A). By contrast, cells grown in the presence of FB1 showed a dramatic inhibition of the formation of BC in a dose-dependent manner, resulting in a reduction of the number of polarized cells by  $\sim$ 75% 2 d after plating (Figure 2, A and B). The inhibitory effect of FB1 on BC formation was reversible. Thus, after 24 h of culturing in the presence of FB1 and subsequent removal of the compound, polarity development was rapidly restored (Figure 2A, dashed line). With the concentrations of FB1 used, we did not observe significant effects on cell viability (trypan blue exclusion; unpublished data), or overall morphology, including cell attachment and spreading. Importantly, the addition of 0.5–2.0  $\mu$ M sphinganine upon plating the cells inhibited HepG2 cell polarity development in a dose-dependent manner (Figure 2C), thereby closely mimicking the effect of FB1 (Figure 2A). Addition of 0.5  $\mu$ M sphinganine for 24 h resulted in an increase in cellular sphinganine levels by  $>$ 20-fold (from  $\sim$ 4.0–90.0 pmol/mg cell protein; Table 2). Similar to as with FB1, cell polarity was rapidly restored upon removal of the exogenous sphinganine (Figure 2C, dashed line). These data suggest that the accumulation of sphinganine, rather than the inhibition of de novo synthesis of ceramides and/or complex sphingolipids, was responsible for the observed impediment of cell polarity development. Indeed, the inhibitory effect of FB1 on polarity development could not be restored when FB1-treated cells were replenished with 10  $\mu$ M C<sub>6</sub>-DHC (Figure 2D) or short-chain ceramides (unpublished data), analogs that were previously shown to restore FB1-mediated effects due to decreased levels of complex sphingolipids (Puglielli *et al.*, 2003).

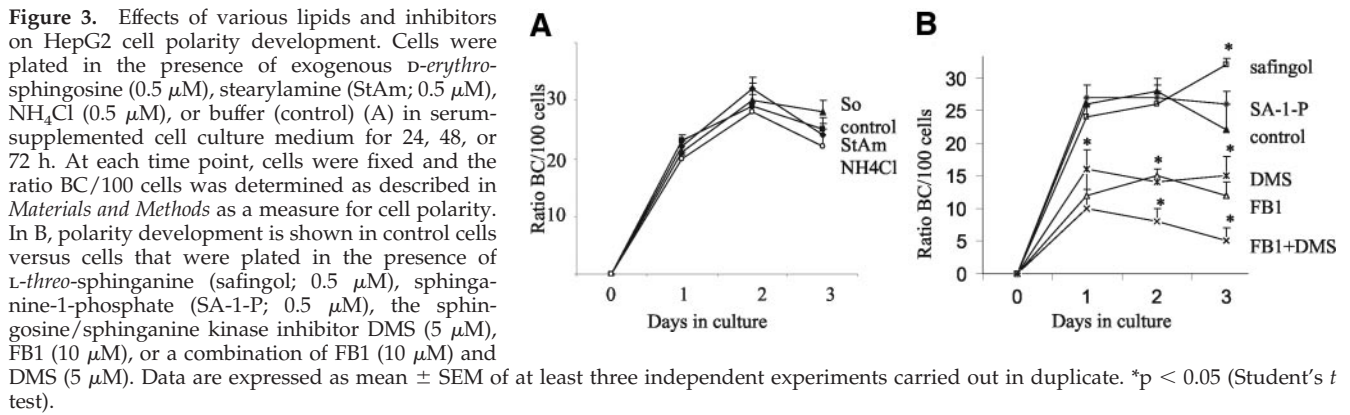
It should be noted that long-term incubation of 3T3 fibroblasts with FB1 was reported to up-regulate the activity of enzymes that produce glucosylceramide and sphingomyelin (Meivar-Levy and Futerman, 1999), both of which have been implicated in differentiation and maturation of specialized plasma membranes in neurons (Futerman *et al.*, 1999; Ledesma *et al.*, 1999). Treatment of HepG2 cells with FB1 under our conditions, however, did not up-regulate glu-

cosylceramide synthase or sphingomyelin synthase (unpublished data). Furthermore, although treatment of cells with FB1 predominantly increases sphinganine levels (Table 1), it has been reported to modestly increase cellular levels of sphingosine (dihydro-sphinganine; Lieser *et al.*, 2003). In HepG2 cells, however, treatment of cells with FB1 (10  $\mu$ M) resulted in a decrease in sphingosine levels; Table 1). Moreover, the exogenous addition of sphingosine did not alter polarity development of HepG2 cells (Figure 3A), in striking contrast to the exogenous addition of sphinganine (Figure 2C). Importantly, the exogenous addition of equal concentrations of hydrophobic amines NH<sub>4</sub>Cl and stearylamine did not affect polarity development (Figure 3A), indicating that the exogenously administered sphinganine did not inhibit polarity development via its potential activity as a hydrophobic amine. In addition, the nonnatural stereoisomer of

**Table 2.** Quantification of cellular sphinganine levels by ESI-MS/MS

	Sphinganine (% of control)
Control	100 (= 4.1 pmol/mg protein)
FB1	561
Exogenous SA	2219
LCS	73
DhcAMP	37

Quantification of sphinganine levels in HepG2 cells. Cells were cultured in normal culture medium in the presence of 10  $\mu$ M FB1, 0.5  $\mu$ M exogenous *D-erythro*-sphinganine (SA), 250  $\mu$ M L-LCS, or 100  $\mu$ M dbcAMP for 24 h. Cells were then lysed in 0.5% SDS lysis buffer, and lysates were immediately frozen at  $-20^{\circ}$ C. The protein content of each sample was determined and sphinganine levels were quantified by ESI-MS/MS analysis as described in *Materials and Methods*. Data were calculated as mean  $\pm$  SEM picomoles per milligram of total cell protein of triplicate experiments ( $n$  = 3) and expressed as percentage of control (4.1 pmol/mg protein; set to 100).



sphinganine, *L-threo*-sphinganine (safingol) did not mimic the effects of FB1 or exogenous (*D-erythro*-)sphinganine (Figure 3A).

Together, the data indicate that dihydroceramide synthase inhibition and subsequent accumulation of *D-erythro*-sphinganine inhibits the establishment of HepG2 cell polarity.

#### *Sphinganine and Not Sphinganine-1-Phosphate Interferes with Polarity Development*

On its accumulation, free sphinganine can be phosphorylated by sphingosine/sphinganine kinase to form sphinganine-1-phosphate. Phosphorylated sphingoid bases are highly bioactive compounds that can elicit various signaling cascades in cells. To determine whether the observed effect of FB1 or exogenous sphinganine on cell polarity development was the result of the phosphorylated product, cells were incubated with FB1 in the presence of *N,N*-dimethylsphingosine (DMS; 2–5  $\mu$ M), an inhibitor of sphingosine/sphinganine kinase (Figure 1). As shown in Figure 3B, DMS did not prevent the effects of FB1 on polarity development. Rather, inhibition of sphinganine kinase activity augmented the inhibitory effect of FB1 on polarity development, and treatment of the cells with DMS alone inhibited polarity development to a similar extent as FB1 (Figure 3B). In addition, treatment of the cells with *D-erythro*-sphinganine-1-phosphate (5  $\mu$ M) did not mimic the effects of sphinganine (Figure 3B). Together, these data suggest that the accumulation of sphinganine, and not of sphinganine-1-phosphate, perturbs polarity development in HepG2 cells.

#### *Sphinganine Turnover Promotes Polarity Development*

To obtain further support for a direct correlation between sphinganine level and cell polarization, we next investigated whether decreased levels of this sphingolipid metabolite also affect cell polarity development. A specific inhibitor of serine palmitoyltransferase, *L*-cycloserine (LCS), inhibits sphinganine biosynthesis (Figure 1). Indeed, the treatment of HepG2 cells with 250  $\mu$ M LCS, in contrast to FB1, resulted in a reduction of the cellular sphinganine level with ~27% (Table 2). Interestingly, in striking contrast to the effect of FB1, LCS treatment enhanced cell polarity development, in terms of both kinetics and maximum polarity, as evidenced by the steep increase of the number (Figure 4A), as well as the size (Figure 4B) of the BCs. Similar results were obtained with another specific inhibitor of serine palmitoyltransferase, ISP-1/myriocin (unpublished data; c.f. Figure 4). The opposing effects of LCS and FB1 on polarity development, under the experimental conditions used, point to sphinganine turnover, rather than to decreased levels of (dihydro)

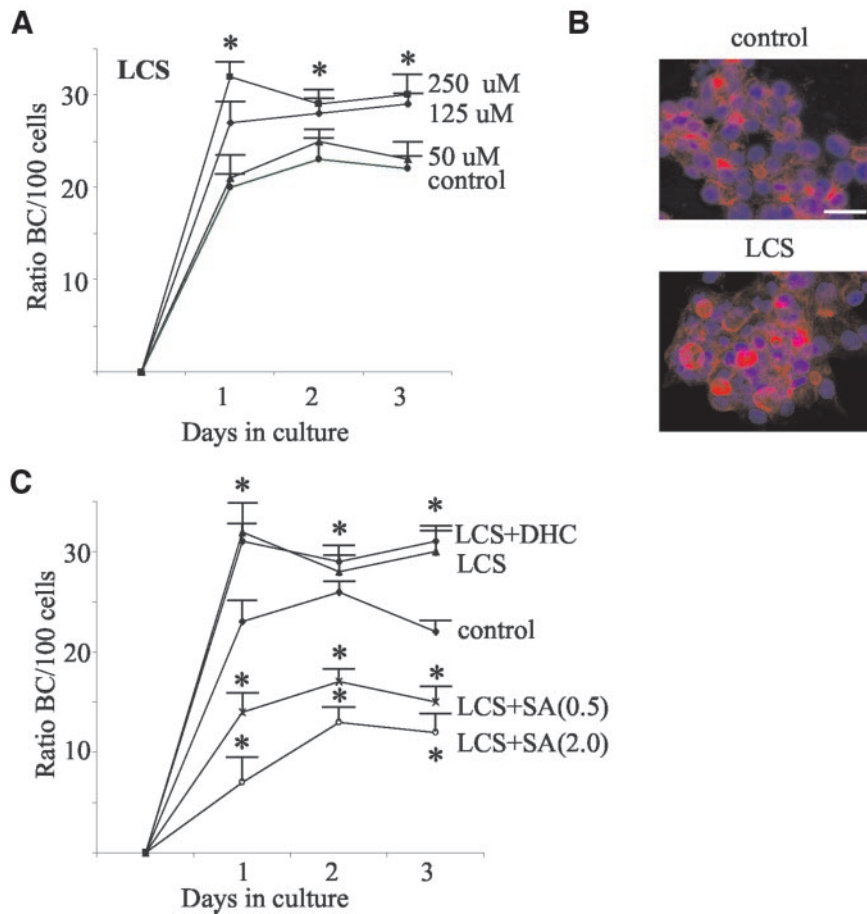
ceramide and/or complex sphingolipids as a crucial parameter in polarity development, a decrease promoting and an increase inhibiting polarity development. Indeed, the polarity-stimulating effect of LCS treatment was completely abolished in the presence of increasing concentrations of exogenous sphinganine (Figure 4C), whereas exogenous  $\text{C}_6$ -DHC (Figure 4C) or ceramide (unpublished data) were without effect.

#### *Dihydroceramide Synthase Is a Target for Cell Polarity-Stimulating Signaling Cascades*

Because the data strongly indicate that the establishment of HepG2 cell polarity is critically dependent on the level of sphinganine, we next investigated whether dihydroceramide synthase, the predominant enzyme in sphinganine turnover, functions as a target for cellular signals that contribute to polarity development. For this, we took into account our previous observation that the stable cAMP analog dbcAMP, via activation of PKA, stimulates HepG2 cell polarity development (Zegers and Hoekstra, 1997; van IJzendoorn and Hoekstra, 1999a, 2000). Importantly, quantification of sphinganine from cell extracts by ESI-MS/MS revealed that the cellular sphinganine level was reduced with ~63% in dbcAMP-treated cells, compared with nontreated cells (Table 2), which is in agreement with the observation that reduced sphinganine levels promote polarity development. To measure the dihydroceramide synthase activity, control and dbcAMP-treated HepG2 cells were homogenized, and incubated with [ $^3\text{H}$ ]sphinganine and stearyl CoA (at saturating concentrations) for 10 min. During this time interval, the activity of DHC synthase was up-regulated ~1.5-fold in dbcAMP-treated cells, as evidenced by the increased production of radiolabeled (dihydro)ceramides ( $22.1 \pm 2.4$ – $32.6 \pm 3.8$  pmol/mg protein), whereas treatment with FB1 blocked the activity of the enzyme (Figure 5A). Importantly, whereas dbcAMP typically stimulates polarity development in HepG2 cells (Figure 5B; c.f. van IJzendoorn and Hoekstra, 1999a, 2000), dbcAMP failed to stimulate polarity development in dihydroceramide synthase-inhibited or sphinganine-treated cells (Figure 5B). We conclude that the reduction of cellular free sphinganine, regulated by signal-mediated modulation of acyl CoA-dependent dihydroceramide synthase activity is a crucial parameter in cAMP/PKA-stimulated HepG2 cell polarity development.

#### *Sphinganine Interferes with Polarized Membrane Traffic from SAC*

Endogenous cAMP/PKA activity is required for HepG2 cell polarity development (van IJzendoorn and Hoekstra, 2000),



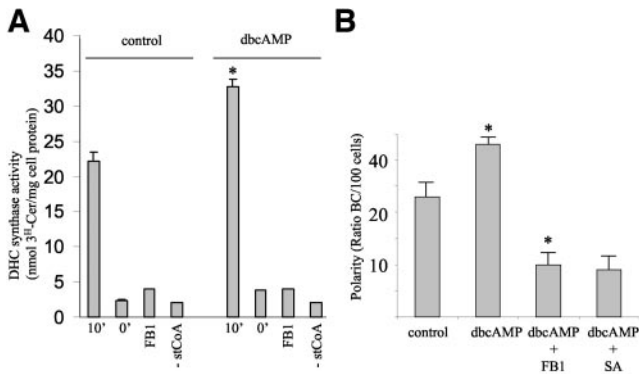
**Figure 4.** LCS promotes HepG2 cell polarity development. Cells were plated in the presence of different concentrations of LCS, LCS + DHC, or LCS + sphinganine in serum-supplemented cell culture medium for 24, 48, or 72 h. At each time point, the cells were fixed, and the ratio BC/100 cells was determined as described in *Materials and Methods*. Effect of 50–250  $\mu$ M LCS (A and B), 250  $\mu$ M LCS + 10  $\mu$ M C<sub>6</sub>-DHC (C), or 250  $\mu$ M LCS + 0.5–2.0  $\mu$ M sphinganine (C) on cell polarity development. Data are expressed as mean  $\pm$  SEM of at least three independent experiments carried out in duplicate. (B) Fluorescence microscopical evaluation of cell polarity in control or LCS-treated cells (72 h; nuclei in blue and BC in red). Note the increase in BC circumference in LCS-treated cells. Bars, 5  $\mu$ m; \* $p$  < 0.05 (Student's *t* test).

and moreover, it mediates the stimulating effects of interleukin-6 family cytokines on BC biogenesis (van der Wouden *et al.*, 2002). We previously identified a specific BC (apical PM)-directed traffic pathway originating from the SAC in HepG2 cells as a primary target for cAMP/PKA signaling (van IJzendoorn and Hoekstra, 1999a; van IJzendoorn *et al.*, 2000). Thus, cAMP/PKA activates a specific SAC-to-BC vesicular traffic route, which is followed by distinct, fluorescently labeled lipids, including sphingomyelin and galactosylceramide (GalCer; Figure 6). Inhibition of this route effectively prohibits polarity development (van IJzendoorn and Hoekstra, 2000). Because the accumulation of sphinganine prevented dbcAMP-stimulated development of HepG2 cell polarity (Figure 2, A and B), we next investigated whether the observed effect was directly correlated with a frustration of cAMP/PKA signaling to redirect and stimulate SAC-to-BC membrane trafficking.

To this end, cells were pretreated with FB1 (10  $\mu$ M), D-erythro-sphinganine (0.5  $\mu$ M), or nothing for 24 h, and SAC was preloaded with fluorescent GalCer as described in *Materials and Methods* (c.f. van IJzendoorn and Hoekstra, 1998, 2000). It is important to note that pretreatment of the cells with FB1 or sphinganine did not affect basolateral internalization, apical delivery, or transport of the fluorescent probe from the apical surface to the SAC. After the loading of the SAC with the fluorescent probe, cells were subsequently treated with dbcAMP (100  $\mu$ M) or buffer at 4°C, warmed to 37°C, and lipid flow from SAC to either apical (BC) or basolateral membrane was followed for 20 min. If appropriate, FB1 or sphinganine were kept present during all incu-

bation steps. In control cells, C<sub>6</sub>NBD-GalCer disappeared during this time interval from the BCP, which constitutes SAC (where the lipid is located at  $t = 0$ ) and BC. Indeed, the percentage of BCP that contained C<sub>6</sub>NBD-GalCer decreased in time (Figure 6A, 1 and 2). The relative distribution of the lipid analogue in the remaining, faintly labeled C<sub>6</sub>NBD-GalCer-positive BCP shows that the probe predominantly resided in SAC and little if any movement from SAC to BC could be detected (Figure 6, B and C, 1 and 2). Rather, these data indicate that the probe was transported from SAC to the basolateral domain of the cells, consistent with previous observations (van IJzendoorn and Hoekstra, 1999, 2000). By contrast, in dbcAMP-treated cells, C<sub>6</sub>NBD-GalCer remained associated with the BCP region during the 20-min chase, as evidenced by the unchanged percentage of BCP that contained C<sub>6</sub>NBD-GalCer (Figure 6A, 1 and 3). Analysis of the corresponding relative distribution of the probe in the BCP reveals a flow of the lipid analogue from SAC to BC (Figure 6, B and C, 1 and 3). In striking contrast, dbcAMP failed to target C<sub>6</sub>NBD-GalCer from SAC to BC in cells in which sphinganine turnover was inhibited (Figure 6, B and C, 1 and 4), but *not* in cells pretreated with LCS, in which the sphinganine levels are decreased (unpublished data; c.f. Figure 6, 1 vs. 3). Thus, in cells with elevated sphinganine levels, C<sub>6</sub>NBD-GalCer remained associated with the BCP during the 20-min chase (Figure 6A, 1 and 4), and analysis of the relative distribution of the lipid analogue in the labeled BCP revealed that most C<sub>6</sub>NBD-GalCer localized to SAC (Figure 6, B and C, 1 and 4), indicating entrapment of the lipid in SAC. Identical results were obtained when exoge-



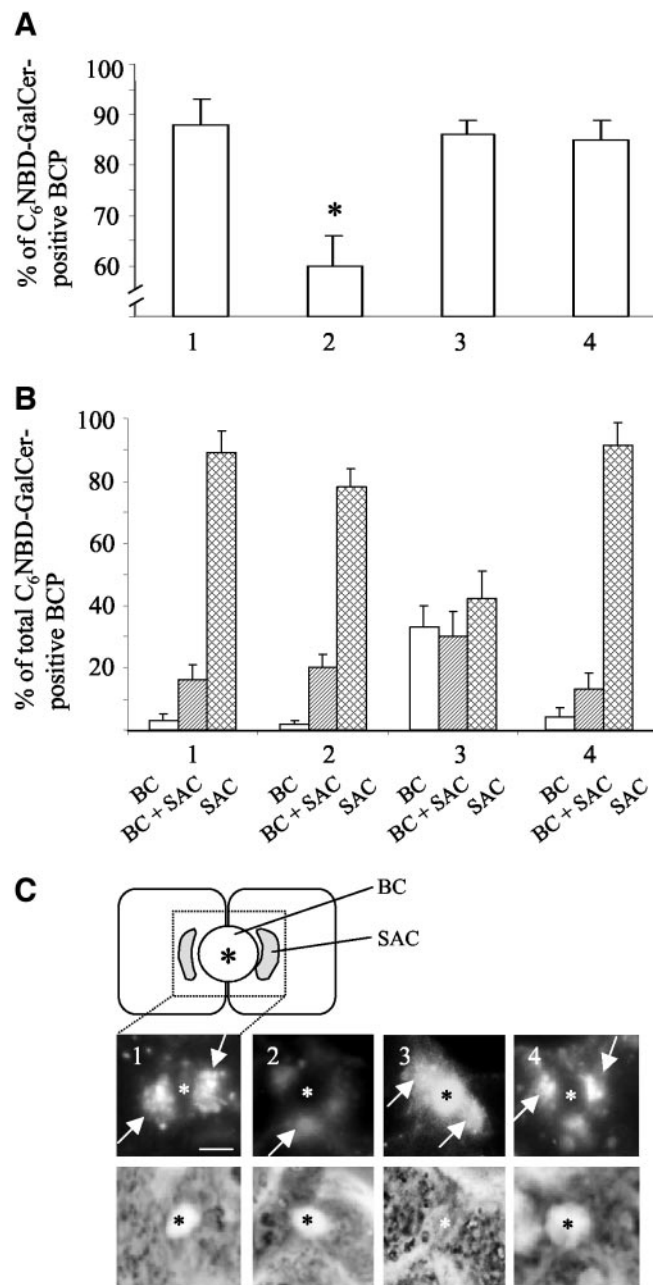


**Figure 5.** (A) dbcAMP stimulates FB1-sensitive acyl-CoA-dependent DHC activity. Control and dbcAMP-treated (0–24 h) HepG2 cells were homogenized and incubated with [ $^3$ H]sphinganine (100 pmol/100  $\mu$ g of cell protein) and stearyl-CoA (10 nmol/100  $\mu$ g of cell protein) for 10 or 0 min at 37°C. Lipids were then extracted with  $\text{CHCl}_3$ :methanol and separated by TLC. Radiolabeled sphinganine and (dihydro)ceramides were scraped from the TLC plates and quantified in a liquid scintillation counter (Veldman *et al.*, 1998). Note that as negative controls, the omission of stearyl (St)-CoA from the reaction or cotreatment of the cells with FB1 prevented dihydroceramide synthase activity. (B) Increased levels of free sphinganine block dbcAMP-stimulated polarity development in HepG2 cells. Cells were cultured in the presence of dbcAMP, dbcAMP + FB1 (10  $\mu$ M), or dbcAMP + exogenous sphinganine (SA; 0.5  $\mu$ M) in serum-supplemented culture medium for 24 h. Cells were then fixed and the ratio BC/100 cells was determined as described in *Materials and Methods*. Note the increase in polarity in dbcAMP-treated cells and the decrease in polarity development in cells treated with dbcAMP together with FB1 or exogenous sphinganine, compared with untreated control cells. Data are expressed as mean  $\pm$  SEM of at least three independent experiments carried out in duplicate.

nous sphinganine was added to the cells during the 4°C dbcAMP treatment before the chase (unpublished data). These data strongly suggest that dihydroceramide synthase activity and the subsequent turnover of sphinganine levels is a prerequisite for cAMP/PKA signaling to redirect polarized membrane traffic from SAC, and in this way, to stimulate polarity development.

#### Sphinganine Inhibits Transcytosis of dIgA-pIgR at the SAC

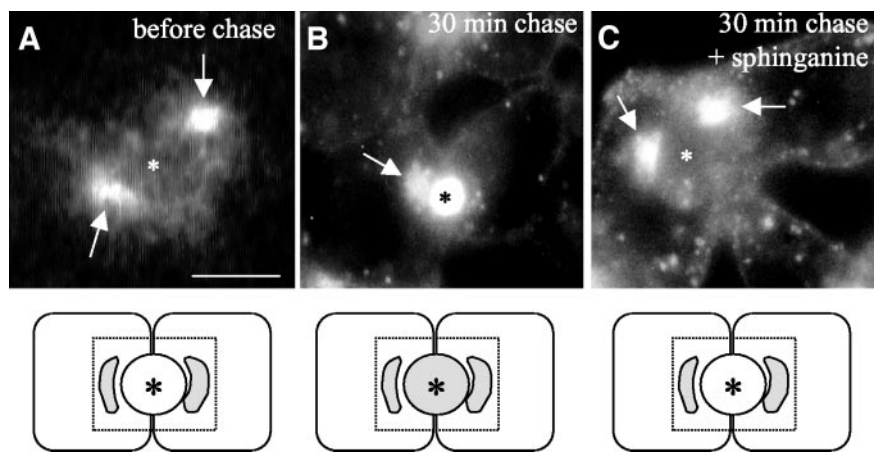
Many apical PM proteins reach the apical surface via an indirect route that includes initial targeting from the Golgi apparatus to the basolateral surface, followed by transcytosis to the apical domain. The transcytotic pathway involves passage through distinct compartments. Thus, after basolateral endocytosis, proteins are first delivered to basolateral early endosomes. Apical PM proteins are then transported to the SAC, and subsequently, to the apical surface (Rojas and Apodaca, 2002; Tuma and Hubbard, 2003). We have previously shown that the cAMP/PKA-activated SAC-to-apical membrane traffic pathway also is followed by the transcytosing dIgA-bound polymeric Ig receptor (pIgR; van IJzendoorn and Hoekstra, 1998) and may thus represent the final leg of the transcytotic pathway. To investigate whether accumulation of sphinganine also interfered with dIgA-pIgR exit from the SAC, HepG2 cells that stably express pIgR (van IJzendoorn and Hoekstra, 1998) were treated with exogenous D-erythro-sphinganine (0.5  $\mu$ M) or buffer (control) for 30 min at 37°C and incubated with Texas-Red-labeled dIgA (50  $\mu$ g/ml) at 18°C for 60 min. This allowed the dIgA-pIgR



**Figure 6.** Accumulation of sphinganine interferes with polarized membrane trafficking from SAC. SAC of control (A–C, 1–3) or FB1-treated (24 h; A–C, 4) HepG2 cells were preloaded with  $\text{C}_6$ -NBD-GalCer, as described in detail previously (van IJzendoorn and Hoekstra, 1998, 2000). After a subsequent chase, the percentage of  $\text{C}_6$ -NBD-GalCer-positive BCP, comprising BC and/or SAC, was determined (A), as well as the corresponding relative distribution of the fluorescent probe in the BCP, i.e., in BC, SAC, or both (BC + SAC) (B). 1. Before the chase ( $t = 0$ ); 2. 20-min chase in HBSS (control cells); 3. 20-min chase in HBSS + 1 mM dbcAMP (control cells); 4. 20-min chase in HBSS + 1 mM dbcAMP (FB1-treated cells). Data are expressed as mean  $\pm$  SEM of at least three independent experiments carried out in duplicate. (C) Representative images showing the distribution of the fluorescent probe in the BCP area marked by the dashed rectangle (i.e., BC alone, BC + SAC, or SAC alone). Similar results were obtained when cells were treated with exogenous sphinganine (0.5  $\mu$ M; 30 min or 24 h) instead of FB1, but not in cells treated with L-threo-sphinganine, sphingosine, or sphinganine-1-phosphate (all 0.5–2.0  $\mu$ M; unpublished data). Asterisks, BC; arrows, SAC. Bottom panels are phase contrast images to top panels. Bars, 5  $\mu$ m.



**Figure 7.** Sphinganine inhibits transcytosis of dIgA-pIgR. HepG2 cells that stably express pIgR were treated with 2.0  $\mu$ M sphinganine (C) or buffer (A and B) for 30 min at 37°C and incubated with Texas Red-labeled dIgA at 18°C for 60 min to accumulate the ligand in SAC (A). Cells were subsequently shifted to 37°C and incubated for another 30 min in buffer in the absence (B) or presence (C) of 0.5  $\mu$ M sphinganine. Note that most of the dIgA-pIgR associated with SAC (A, arrows) is readily transported to the BC (B, asterisk), whereas in cells treated with exogenous sphinganine, dIgA-pIgR is not transported to the BC and is retained in SAC (C, arrows; see text). Treatment of the cells with sphingosine, *L-threo*-sphinganine, or sphinganine-1-phosphate for 30 min at 37°C gave similar results as observed in control cells (unpublished data; c.f. B). Bars, 5  $\mu$ m.



to be internalized and transported to the SAC. Exit from the SAC, however, is greatly impaired at 18°C (Figure 7A; cf. van IJzendoorn and Hoekstra, 1998). Treatment of the cells with sphinganine did not affect basolateral endocytosis and transport to SAC (Figure 7A). Then, cells were warmed to 37°C and dIgA-pIgR was chased for another 30 min in buffer, supplemented or not with sphinganine. In control cells, dIgA-pIgR moved from the SAC to the apical PM domain (Figure 7B), clearly marking the BC circumference, in agreement with our previous data (van IJzendoorn and Hoekstra, 1998). In striking contrast, in sphinganine-treated cells, transport of dIgA-pIgR from the SAC to the apical surface was blocked and the protein accumulated in the SAC (Figure 7C). Similar results were obtained when cells were treated with FB1 (10  $\mu$ M) or DMS (5  $\mu$ M), whereas treatment with sphingosine, *L-threo*-sphinganine or sphinganine-1-phosphate did not mimic the effects of sphinganine on dIgA-pIgR transport (unpublished data). These data indicate that accumulation of sphinganine frustrates the final leg in the transcytotic pathway, i.e., trafficking from SAC to the apical PM domain.

## DISCUSSION

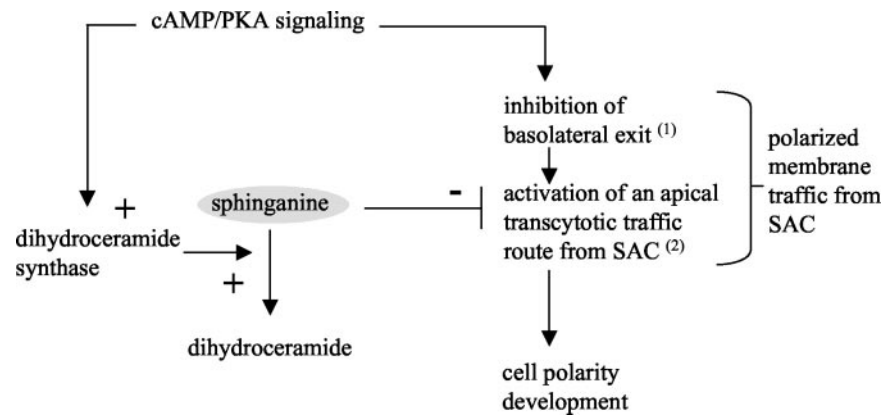
In this study, we implicate regulated sphinganine metabolism as a prominent parameter in cell polarity development. The most direct evidence is provided by the observation that elevated levels of sphinganine, generated either by inhibiting dihydroceramide synthase (sphinganine *N*-acyltransferase), by inhibiting sphinganine kinase, or by administering exogenous *D-erythro*-, but not *L-threo*-sphinganine, effectively and reversibly perturb the *de novo* biogenesis of apical PM domains in well differentiated HepG2 cells (Figures 2 and 3). Moreover, cAMP/PKA signaling cascades that stimulate cell polarity development significantly increase the activity of dihydroceramide synthase (Figure 5), and subsequently, sphinganine turnover, which results in reduced cellular sphinganine levels (Table 2). Importantly, FB1-mediated inhibition of dihydroceramide synthase activity effectively blocked the stimulatory effect of cAMP/PKA signaling on polarity development. The molecular mechanism by which cAMP/PKA signaling increases dihydroceramide synthase activity remains to be investigated. Possibly, cAMP/PKA directly phosphorylates dihydroceramide synthase, or alternatively, it may affect its localization, as demonstrated for other endoplasmic reticulum/Golgi enzymes,

thus affecting substrate clearance. To our knowledge, this is the first report of regulated dihydroceramide synthase activity in response to signaling factors.

Inhibition of dihydroceramide synthase with FB1 resulted in a decrease in ceramides, sphingomyelin, and glycosphingolipids on the one hand, and an increase in cellular sphinganine levels on the other. Because (glyco)sphingolipids have been implicated in cell differentiation and the biogenesis of specialized PM domains in neurons and epithelial cells, we initially thought that the reduction of cellular (glyco)sphingolipids in FB1-treated cells was responsible for the inhibition of polarity development. However, the opposing effects of FB1 and LCS treatment on polarity development, both of which result in decreased levels of higher sphingolipids but have opposite effects on sphinganine levels (Table 2), strongly suggest that the cellular levels of sphinganine, rather than the reduced levels of higher sphingolipids mediated the effect of dihydroceramide synthase activity modulation on polarity development. This is supported by the observation that exogenous addition of *D-erythro*-, but not *L-threo*-sphinganine, closely mimicked the effect of the dihydroceramide synthase inhibitor FB1 in a dose-dependent manner and that the replenishment of ceramides failed to restore the inhibitory effects of FB1 (Figure 2). Because inhibition of sphinganine kinase activity with DMS failed to prevent the effects of FB1, and because treatment of the cells with exogenously added sphinganine-1-phosphate did not mimic the effects of exogenously added sphinganine (Figure 3), we conclude that sphinganine, rather than its phosphorylated products, is responsible for the effects described in this study. The profound effects that an altered level of sphinganine has on cell surface dynamics may provide for a mechanism that underlies the hepatotoxic effects of fumonisin B1 (Marasas *et al.*, 1995) and explains the role of fumonisins in hepatocellular carcinogenesis (Gelderblom *et al.*, 1988; Spiegel and Merrill, 1996), which arises from the dedifferentiation of mature hepatocytes (Bralet *et al.*, 2002; Gournay *et al.*, 2002).

Polarized membrane trafficking and the plasticity thereof is a crucial factor in cell differentiation (reviewed in Mostov *et al.*, 2003). Sphingolipids and their precursors, ceramides and sphingoid bases, have recently emerged as important, but poorly understood modulators of membrane traffic. In yeast, sphingoid bases are involved in endocytosis (Friant *et al.*, 2000, 2001; Zanolari *et al.*, 2000; deHart *et al.*, 2002). Here, we show that elevated levels of sphinganine dramatically perturb polarized membrane traffic from the SAC (Figures 6 and 7), a sub-

**Figure 8.** Working model that describes the relationship between sphinganine metabolism, polarized membrane traffic from SAC, and apical PM biogenesis in response to cAMP/PKA signaling in HepG2 cells. First, cAMP/PKA signaling reorganizes polarized membrane traffic from the SAC, which includes the rerouting of a fluorescent lipid probe from a basolateral direction into the transcytotic pathway, leading to the apical surface. Activation of this pathway is crucial for cAMP/PKA-stimulated polarity development. cAMP/PKA signaling also increases the activity of dihydroceramide synthase, thereby stimulating the turnover of sphinganine. The inability to turn over sphinganine and the consequent accumulation of free sphinganine inhibits cAMP/PKA-induced activation of the apical PM directed traffic route from SAC and, hence, polarity development.



apical endosomal compartment in epithelial cells that plays a prominent role in polarized PM recycling and the biogenesis and dynamics of apical PM domains in hepatocytes (Ihrke *et al.*, 1998; van IJzendoorn and Hoekstra, 1998, 1999a,b, 2000). Thus, cAMP/PKA signaling precludes the exit of C<sub>6</sub>-NBD-GalCer from SAC in a basolateral PM-directed route and activates its targeting to the apical PM domain (Figure 6). This reorganization of polarized membrane traffic from SAC was previously shown to be a crucial parameter in cell polarity development in HepG2 cells (van IJzendoorn and Hoekstra, 1999b, 2000). Conditions that promote sphinganine accumulation, i.e., dihydroceramide synthase inhibition or the exogenous addition of sphinganine, but not the exogenous addition of the nonnatural L-threo-sphinganine or sphinganine-1-phosphate, effectively block the cAMP/PKA-mediated activation of the SAC-to-BC pathway (see model in Figure 8). Exit of the lipid analogue from SAC in a basolateral direction also is precluded, thus resulting in its entrapment in SAC. These data suggest that the cAMP/PKA signaling-stimulated rerouting of C<sub>6</sub>-NBD-GalCer includes two distinct events within SAC. The first step precludes exit from SAC into a vesicular basolateral PM-directed pathway and the second step involves the activation of or entry of the lipid into the SAC-to-BC route. That the exit of the probe into a basolateral PM-directed pathway is precluded also under conditions of accumulated sphinganine suggests that the sphingoid base interferes with membrane traffic from SAC, rather than directly with the cAMP/PKA signaling cascade. Importantly, because SAC was readily loaded with C<sub>6</sub>-NBD-GalCer (Figure 6, A–C, 1), a procedure that involves basolateral endocytosis, apical delivery, and apical-to-SAC transport of the probe (van IJzendoorn and Hoekstra, 1999a, 2000), the effect of sphinganine accumulation on membrane traffic does not seem to reflect a general frustration of all intracellular transport but is rather specific to transport events mediated by SAC. We previously proposed that the SAC-to-BC pathway activated by cAMP/PKA signaling may represent the final leg in the basolateral-to-apical transcytotic route (van IJzendoorn *et al.*, 1999a). The observation that accumulated sphinganine similarly frustrates SAC-to-BC trafficking of transcytosing dIgA-pIgR (Figure 7) supports this hypothesis.

Although our data strongly suggest that elevated levels of sphinganine perturb apical PM biogenesis by frustrating polarized membrane traffic from the SAC, thereby interfering with the involvement of SAC as part of the transcytotic itinerary, the mechanism by which sphinganine affects SAC remains unclear. Sphinganine was reported to perturb endosomal cholesterol transport (Underwood *et al.*, 1996), which results in the accumulation of cholesterol in perinuclear vesicles (Roff *et al.*, 1991; Porpaczy *et al.*, 1997), thereby mimicking the endosomal

accumulation of cholesterol and other (sphingo)lipids in Niemann-Pick Type C (Roff *et al.*, 1991; Rodriguez-Lafrasse *et al.*, 1994) and several sphingolipid storage diseases (Pagano *et al.*, 2000). A potential interference of sphinganine with cholesterol trafficking by virtue of its action as a hydrophobic amine, however, is likely to be excluded, because other hydrophobic amines did not mimic the effects of sphinganine accumulation on HepG2 cell polarity development (Figure 3A). Further studies are needed to clarify the molecular mechanism by which sphinganine interferes with intracellular membrane trafficking.

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